

Original article

Genetic association study of NF- κ B genes in UK Caucasian adult and juvenile onset idiopathic inflammatory myopathy

Hector Chinoy¹, Charles K.-C. Li², Hazel Platt³, Noreen Fertig⁴, Hemlata Varsani⁵, Harsha Gunawardena⁶, Zoe Betteridge⁶, Chester V. Oddis⁴, Neil J. McHugh⁶, Lucy R. Wedderburn⁵, William E. R. Ollier² and Robert G. Cooper^{1,3}, on behalf of the UK Adult Onset Myositis Immunogenetic Consortium and UK Juvenile Dermatomyositis Research Group*

Abstract

Objective. Treatment-resistant muscle wasting is an increasingly recognized problem in idiopathic inflammatory myopathy (IIM). TNF- α is thought to induce muscle catabolism via activation of nuclear factor-kappa B (NF- κ B). Several genes share homology with the NF- κ B family of proteins. This study investigated the role of NF- κ B-related genes in disease susceptibility in UK Caucasian IIM.

Methods. Data from 362 IIM cases [274 adults, 49 (\pm 14.0) years, 72% female; 88 juveniles, 6 (\pm 3.6) years, 73% female) were compared with 307 randomly selected Caucasian controls. DNA was genotyped for 63 single nucleotide polymorphisms (SNPs) from NF- κ B-related genes. Data were stratified by IIM subgroup/serotype.

Results. A significant allele association was observed in the overall IIM group vs controls for the *IKBL*-62T allele (rs2071592, odds ratio 1.5, 95% CI 1.21, 1.89, corrected $P=0.0086$), which strengthened after stratification by anti-Jo-1 or -PM-Scl antibodies. Genotype analysis revealed an increase for the AT genotype in cases under a dominant model. No other SNP was associated in the overall IIM group. Strong pairwise linkage disequilibrium was noted between *IKBL*-62T, *TNF*-308A and *HLA-B*08* ($D' = 1$). Using multivariate regression, the *IKBL*-62T IIM association was lost after adjustment for *TNF*-308A or *HLA-B*08*.

Conclusion. An association was noted between *IKBL*-62T and IIM, with increased risk noted in anti-Jo-1 and -PM-Scl antibody-positive patients. However, the *IKBL*-62T association is dependent on *TNF*-308A and *HLA-B*08*, due to strong shared linkage disequilibrium between these alleles. After adjustment of the 8.1 HLA haplotype, NF- κ B genes therefore do not independently confer susceptibility in IIM.

Key words: polymyositis, dermatomyositis, single nucleotide polymorphisms, immunogenetics, autoantibodies, NF- κ B, TNF.

¹Rheumatic Diseases Centre, Manchester Academic Health Science Centre, The University of Manchester, Salford Royal NHS Foundation Trust, Salford, ²Department of Rheumatology, Royal Surrey County Hospital NHS Foundation Trust, ³Centre for Integrated Genomic Medical Research, Manchester Academic Health Science Centre, The University of Manchester, Manchester, UK, ⁴Division of Rheumatology and Clinical Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA, ⁵Rheumatology Unit, Institute of Child Health, UCL, London and ⁶Rheumatology Department, Royal National Hospital for Rheumatic Diseases, Bath, UK.

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Correspondence to: Robert G. Cooper, The University of Manchester Rheumatic Diseases Centre, Hope Hospital, Salford M6 8HD, UK. E-mail: robert.g.cooper@manchester.ac.uk

*See supplementary data at *Rheumatology* Online for members of the UK Juvenile Dermatomyositis Research Group who contributed to this work.

Introduction

The idiopathic inflammatory myopathies (IIMs) represent a group of autoimmune muscle diseases characterized by muscle weakness, inflammatory muscle infiltrates in muscle biopsy samples and electromyographic abnormalities on neurophysiological testing. Although mortality rates are reportedly low [1, 2], IIM patients can suffer considerable disease-related morbidity or treatment-related complications. Thus, patients may become significantly disabled, with progressive weakness. Adults with IIM may suffer irreversible muscle wasting despite treatment and apparent resolution of inflammatory cell infiltrates [3]. Juvenile cases may additionally suffer specific complications including ongoing skin disease such as ulceration, calcinosis and joint contractures.

The cause of this irreversible muscle atrophy in adult IIM is unknown, although it is speculated that disease-related alteration of the endoplasmic reticulum stress response may cause metabolic changes to energy metabolism and fibre dysfunction [4]. The pro-inflammatory cytokine, TNF- α , is involved in muscle protein catabolic processes. TNF- α is thought to induce protein loss via oxidative activation of the myogenic transcription factor nuclear factor-kappa B (NF- κ B). Furthermore, NF- κ B p50 and p65 have been described within the inflammatory exudates of different IIM subtypes [5]. Several genes have been identified that share homology with the NF- κ B family of proteins, including *NFKB1*, *NFKB1A*, *NFKB1B*, *NFKB1E*, *IKBL* (*NFKBIL1*), *REL*, *RELB* and *BCL3*. Current evidence for genetic risk in IIM arises from candidate gene studies comparing cases with controls. In a manner similar to other autoimmune diseases, the main IIM genetic risk factors lie within the HLA region, notably with components of the 8.1 common ancestral haplotype (8.1 haplotype) (HLA-B*08/DRB1*03/DQB1*02/DQA1*05) especially in the presence of certain myositis-specific/associated antibodies (MSAs/MAAs) [6–10]. Non-HLA genes are increasingly recognized as conferring a degree of risk [11]. In view of the apparently key role that NF- κ B may play in skeletal muscle protein catabolism and the described clinical problem of muscle dysfunction and wasting in IIM, the study reported here investigated genes involved with the NF- κ B pathway in adult and juvenile UK Caucasian IIM patients.

Patients and methods

Subjects

DNA was available from 362 UK Caucasian IIM cases. Adult IIM patients ($n=274$), aged ≥ 18 years of age at disease onset, were recruited through the UK Adult Onset Myositis Immunogenetic Collaboration (AOMIC) [10]. JDM patients ($n=88$) were recruited via the UK Juvenile Dermatomyositis National (UK and Ireland) Cohort Biomarker Study and Repository [12–14]. Patients with PM, DM or JDM had probable or definite myositis, based on the Bohan and Peter criteria [15, 16]. Myositis/CTD-overlap patients were included if they

fulfilled all of the following: (i) met published criteria for their primary CTD [17–21] or MCTD [22]; (ii) possessed at least two of four Bohan and Peter criteria (proximal muscle weakness, elevated muscle enzymes, characteristic myopathic EMG changes and diagnostic muscle biopsy); and (iii) possessed at least one MSA/MAA. A standardized clinical data collection form detailed demographics and individual clinical details.

Controls

Three hundred and seven UK Caucasian control subjects were recruited from blood donors and general practitioner registers as described [10]. The study was approved by local research ethics committees [Northern and Yorkshire Multi-centre Research Ethics Committee (juvenile cases MREC 1/3/22); North West Research Multi-centre Research Ethics Committee (adult cases MREC 98/8/86)] and full informed consent was obtained according to the Declaration of Helsinki.

Autoantibody typing

Serum was obtained from patients for determination of MSAs: anti-synthetases: -Jo-1, -PL-7, -PL-12, -EJ, -OJ, -KS; anti-Mi-2, anti-SRP, anti-155/140; and MAAs: anti-PM-Scl, anti-Ku, anti-U1-RNP, anti-U3-RNP using radioimmunoprecipitation, as previously described in adult [10, 23] and juvenile IIM [13].

Genotyping

DNA samples were extracted from a peripheral blood sample obtained from both cases and controls using a standard phenol-chloroform method. Single nucleotide polymorphisms (SNPs) were genotyped using the Sequenom MassArray iPLEX platform, as per the manufacturer's instructions (<http://www.sequenom.com/seq-genotyping.html>). HLA Class I and TNF typing have been described previously [10, 24].

NF- κ B SNPs

Sixty-three SNPs within the NF- κ B family were initially selected from the following genes: *NFKB1*, *NFKB1A*, *NFKB1B*, *NFKB1E*, *IKBL* (*NFKBIL1*), *REL*, *RELB* and *BCL3*. Thirty-eight haplotype tagging (ht) SNPs were selected for genotyping using the Hapmap CEU population (release 20, National Center for Biotechnology Information B35 assembly, online at www.ncbi.nlm.nih.gov) by pairwise tagging, r^2 cut-off ≥ 0.8 and a minor allele frequency (MAF) of 10%. The remainder of the SNPs were selected on the basis of being synonymous, non-synonymous, 3'-untranslated region or in the 5'-upstream region. Conserved non-coding SNPs were also selected using an approach outlined by Bejerano *et al.* [25], to obtain sequences of conserved non-coding regions from a wide range of organisms, using the UCSC Genome Browser database (<http://genome.ucsc.edu/cgi-bin/hgGateway>). Fourteen SNPs were removed as they were rejected by Sequenom assay design, three SNPs were removed as they were not compatible with the plexes created and three SNPs were rejected where the

TABLE 1 *IKBL*-62T allele associations in clinical and antibody IIM subgroups

	<i>n</i>	Minor allele (T) frequency (%)	OR (95% CI)	<i>P</i> -value	Bonferroni-corrected <i>P</i> -value
Controls	307	34.4			
Overall	362	44.3	1.5 (1.21, 1.89)	0.0002	0.0086
PM	112	44.6	1.5 (1.12, 2.10)	0.004	0.17
DM	98	42.9	1.4 (1.03, 1.99)	0.03	1.29
CTD/overlap	64	47.7	1.7 (1.18, 2.55)	0.006	0.26
JDM	88	42.6	1.4 (1.01, 2.0)	0.05	2.15
Jo-1	50	58.0	2.6 (1.72, 4.05)	0.00001	0.0004
PM-Scl	36	59.7	2.8 (1.72, 4.65)	0.00005	0.002

Significant antibody associations are shown in bold. Associations are presented assuming a dominant mode of inheritance.

sample genotyping success rate was <85%. For the purposes of analysis, assay success cut-off was set at 90%. Thus, a remainder of 43 SNPs were available for the final analysis (a summary of the SNPs is available in the supplementary Table 1, available as supplementary data at *Rheumatology* online). A power calculation was applied to the SNP with the lowest MAF (rs4648127, 10%). For 95% power to detect an effect size of 2.0 at a 95% significance level, a sample size of 338 cases and 282 controls would be required.

Statistical analyses

Genotype frequencies for each NF- κ B SNP were tested for Hardy-Weinberg equilibrium (HWE) in each group. Allele and genotype frequencies of these NF- κ B SNPs were compared between myositis cases and controls, using Fisher's exact test or chi-square test, as appropriate. Where significant, data were expressed as odds ratios (ORs) with exact 95% CIs and pointwise *P*-values were corrected using Bonferroni. Linkage disequilibrium (LD) was calculated using the measure of *D'*. The analyses were also repeated after stratification by clinical and serological subgroup. Unless otherwise stated, the statistical package Stata (release 9.2, Stata Corp., College Station, TX, USA) was used to perform statistical analysis.

Results

The breakdown of the IIM groups was as follows: 274 adults, 49 (\pm 14.0) years, 72% female; 88 juvenile, 6 (\pm 3.6) years, 73% female. All 43 SNPs conformed to HWE. The frequency of the *IKBL*-62T allele (rs2071592) was significantly increased in the overall IIM group vs controls (Table 1). This association persisted after stratification by clinical subgroup, and no significant difference was observed between the subgroup frequencies of *IKBL*-62T. The data were then further stratified by serological subgroup. The *IKBL*-62T association was strengthened after stratification by either anti-Jo-1 or -PM-Scl antibody (Table 1). Genotype analysis of the overall IIM group revealed an increase of the AT genotype in cases (Table 2), with a significant association noted under a

TABLE 2 *IKBL62* SNP genotype frequencies in combined IIM cases compared with controls

Genotype	Controls (<i>n</i> = 307) <i>n</i> (%)	IIM (<i>n</i> = 362) <i>n</i> (%)
AA	134 (43.6)	101 (27.9)
AT	135 (44.0)	202 (55.8)
TT	38 (12.4)	59 (16.3)

TABLE 3 *IKBL62* SNP genotype associations in overall IIM cases vs controls

Genotype test	<i>P</i>	OR (95% CI)
AT + TT vs AA	0.00002	2.0 (1.45, 2.76)
TT vs AT + AA	0.15	1.38 (0.89, 2.14)
TT vs AA	0.003	2.06 (1.27, 3.34)
AT vs AA	0.88	0.96 (0.61, 1.53)

P-values are uncorrected.

dominant model of inheritance (Table 3). No other SNP was significantly increased in the overall IIM group or clinical/serological subgroups. The relationship between *IKBL*-62T, TNF- α and HLA Class I was then examined. The relationship between *TNF*-308A and HLA-B*08 in adult IIM has previously been described [24]. Strong pairwise LD was noted between *IKBL*-62T, *TNF*-308A and HLA-B*08 (*D'* = 1). Using multivariate logistic regression, the *IKBL*-62T association with IIM was lost after adjusting for the presence of either *TNF*-308A or HLA-B*08.

Discussion

This candidate gene study tested the hypothesis that NF- κ B-related genes may confer susceptibility in IIM. An SNP, rs2071592 (*IKBL*-62T), was found to be

significantly increased in IIM, especially in anti-Jo-1- and PM-Scl-positive cases when compared with controls. However, a strong LD relationship was confirmed between *IKBL*-62T and alleles forming part of the HLA 8.1 ancestral haplotype, and indeed after adjusting for *TNF*-308A and *HLA-B*08*, the association of *IKBL*-62T with IIM was lost.

The *IKBL*-62T SNP implicated in this study is in the promoter region of the *IKBL* gene, which is located on the telomeric end of the MHC on Chromosome 6, and codes for a member of the I κ B family. I κ B proteins have a regulatory role, through binding and then sequestration of NF- κ B in the cytoplasm, thus inhibiting translocation to the nucleus [26]. Polymorphisms within genes encoding I κ B proteins have been investigated in a number of other autoimmune conditions including multiple sclerosis, RA, type 1 diabetes mellitus, SLE and Grave's disease in different populations [27–33].

Data surrounding the *IKBL*-62T SNP remain conflicting. Disruption of an E-box binding element in the *IKBL* promoter by the *IKBL*-62T SNP has been described in binding studies [34], which of interest is a sequence shared in many B- and T-cell lineage-specific genes. Decreased *IKBL* promoter activity has also been functionally associated with the *IKBL*-62A allele, culminating in an exaggerated NF- κ B-mediated inflammatory response [35]. A study of subjects with Grave's disease identified a higher frequency of *IKBL*-62A when compared with healthy controls [33]. In contrast, *IKBL*-62T was found to be linked to RA in a Japanese population study [29], then known as SNP96452 (OR 2.08, $P = 0.0088$). This study did not, however, report on LD effects with *HLA-DRB1* alleles, nor was the effect replicated in a UK family-based study [30] or a Spanish case-control study [31].

There is increasing evidence for a role of the NF- κ B in alteration of normal muscle physiology. Transgenic overexpression of NF- κ B (via activated I κ B kinase b) causes profound muscle wasting in mice via accelerated protein breakdown through ubiquitin-dependent proteolysis [36]. In a mechanistically reverse experiment, Mourkioti *et al.* [37] demonstrated that suppression of NF- κ B response by *IKK2* muscle-specific deletion protected skeletal muscle from atrophy despite denervation injury via both enhanced regeneration (satellite cell activation) and reduced fibrosis. In human IIM, NF- κ B activation has been demonstrated by western blot analysis and electrophoretic mobility shift assays on muscle biopsies in patients with PM and DM [38]. Furthermore, NF- κ B p50 and p65 is found in CD4⁺ T cells in muscle biopsies from both PM and IBM [5]. In human IBM and a myositis mouse model, overexpression of MHC Class I on muscle fibres results in activation of NF- κ B and an endoplasmic reticulum stress response [4, 39, 40]. LEF, among its many actions, is a potent NF- κ B inhibitor and has been reported to be effective in resistant cases of PM and DM [41, 42].

Alleles forming part of the 8.1 Caucasian MHC common ancestral haplotype (*HLA-A1-B8-Cw7-DRB1*0301-DQA1*0501-C4A*Q0*) occur in strong LD within Caucasian populations in northern and western Europe,

and represent risk factors for a large number of immunopathological diseases [43]. To date, the 8.1 haplotype has also been identified as a major risk factor in IIM [44, 45]. Thus, *IKBL*-62T confers risk for IIM as this allele forms part of the 8.1 haplotype where it shares strong LD with other alleles. We hypothesize that an altered NF- κ B response and cytokine profile could relate to previous observations that the 8.1 haplotype confers an immune hyperresponsiveness [46, 47].

Due to the rarity of IIM, difficulties will always be encountered when trying to recruit a sufficient number of cases for analysis in genetic association studies that examine SNPs with a modest effect size. The present study was not powered to detect associations after stratification by disease or serological subgroups. This may also explain why no significant associations were observed for the other SNPs with a low MAF. The SNPs tested were htSNPs, which means that the true causal SNP may not have been tested directly in the current study. Due to the limited number of SNPs tested and assay design failure, there will be incomplete coverage of the tested regions. Furthermore, we have not corroborated our findings with functional data. Finally, clinical associations with these SNPs may be due to factors other than irreversible muscle atrophy, certainly within the juvenile cohort where this clinical feature is generally not typical.

To conclude, these findings describe the association of *IKBL*-62T with IIM susceptibility in a large cohort of adult and juvenile UK Caucasians. Significant differences are not apparent between IIM subgroups and the risk appears strongest in anti-Jo-1 and -PM-Scl cases, where the 8.1 haplotype association is the strongest. This study furthers our understanding of the 8.1 haplotype in IIM and should trigger off more detailed and functional studies into NF- κ B-related pathways.

Rheumatology key messages

- An association is noted between *IKBL*-62T and IIM, and increased risk in Jo-1/PM-Scl-positive patients.
- The *IKBL*-62T association is dependent on *TNF*-308A and *HLA-B*08* due to strong shared LD.
- NF- κ B genes do not confer susceptibility in IIM after adjustment of the 8.1 HLA haplotype.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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